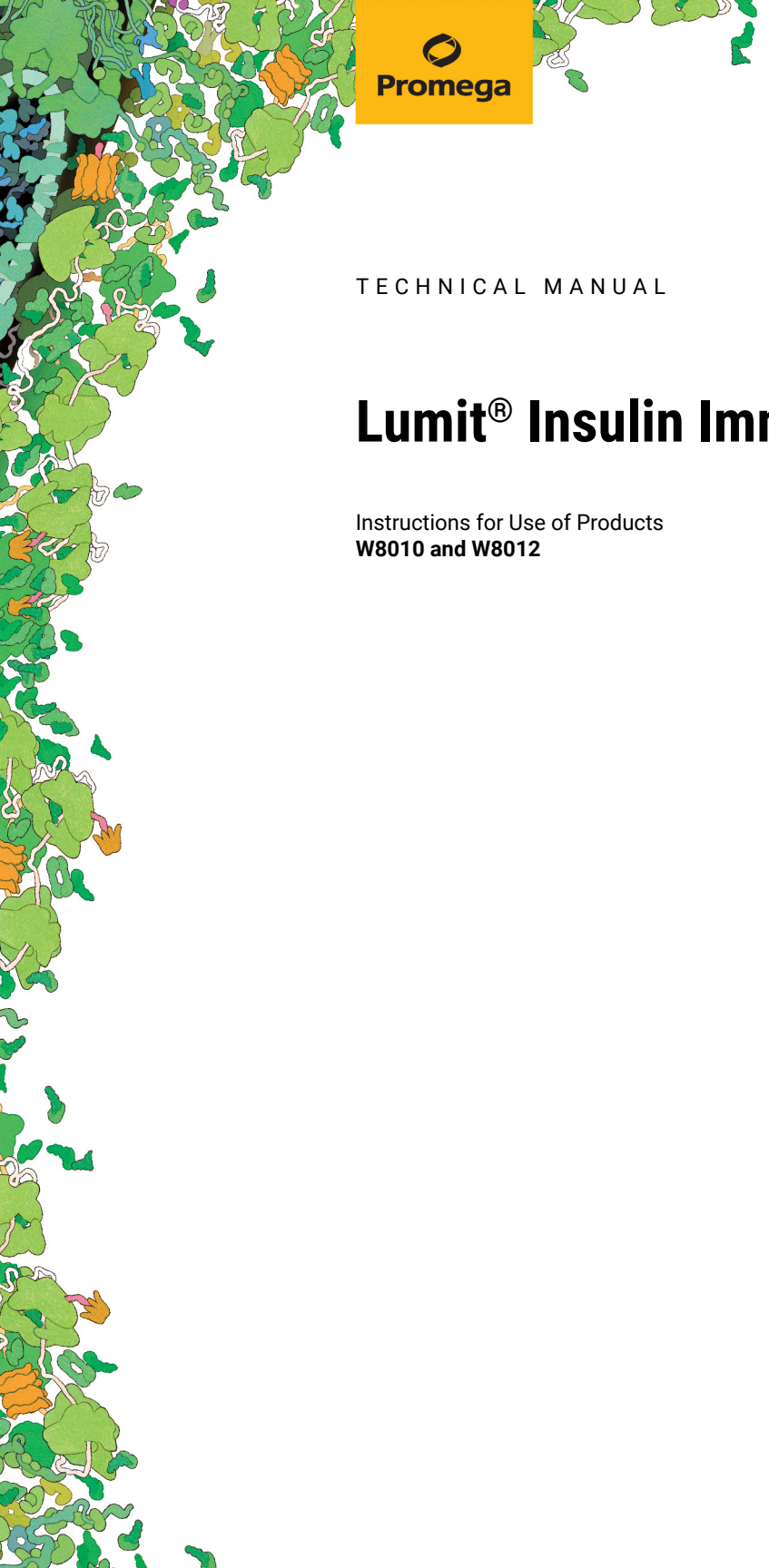

TECHNICAL MANUAL

Lumit® Insulin Immunoassay

Instructions for Use of Products
W8010 and W8012

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TM776

Promega

Lumit® Insulin Immunoassay

All technical literature is available at: www.promega.com/protocols/
Visit the website to verify that you are using the most current version of this Technical Manual.
Email Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The Lumit® Insulin Immunoassay[a,b] is a homogeneous, bioluminescent assay for detecting insulin in solution without the need for wash steps. This immunoassay has been developed for use with cell culture samples to measure insulin secreted from cell lines or islets. The assay has a range of 10pM–8nM insulin and is compatible with samples from human, mouse and rat cells and isolated islets. (See representative data in Section 6.)

Insulin is the key regulator of glucose homeostasis in the body. It is a small, 51 amino acid, peptide hormone that is secreted from beta cells present in the islets of Langerhans of the pancreas. It is processed from proinsulin in cytoplasmic secretory granules where it is stored until released in response to glucose (1). Once released, it signals uptake of circulating glucose by tissues and promotes glucose storage in the liver through glycogen synthesis. The inability to make insulin or to respond to insulin (insulin resistance) leads to abnormal carbohydrate metabolism and diabetes (2).

Assay Principle

The Lumit® Insulin Immunoassay is based on NanoLuc® Binary Technology (NanoBiT). NanoBiT is a luminescent structural complementation system designed for biomolecular interaction studies (3,4). The system is composed of two subunits, Large BiT (LgBiT; 18kDa) and Small BiT (SmBiT; 11 amino acid peptide), that were optimized for stability and minimal spontaneous association. In this assay, a sample is incubated with a pair of anti-insulin monoclonal antibodies covalently labeled with SmBiT or LgBiT. When the labeled antibodies bind to insulin, the complementary LgBiT and SmBiT are brought into proximity, thereby reconstituting NanoBiT® enzyme and generating luminescence in the presence of the Lumit® substrate. The luminescent signal is directly proportional to the amount of insulin present in the sample (Figure 1).

Assay Format

The no-wash, in-solution protocol for this immunoassay (Figure 2) offers flexibility in terms of both the number of data points that can be assayed in an experiment and the volume of sample that can be used. It is compatible with various multiwell formats (e.g., 96- and 384-well) and multiple sample volumes (e.g., 5–100µl).

Customize the assay format to your needs, provided you maintain the ratio of sample to reagent volume. The number of data points that can be collected using several sample volumes are listed in Table 1. For example, one kit (Cat.# W8010, 100–400 assays) contains sufficient reagents for 100 assays in 96-well plates (50µl sample volume) or 400 assays in 384-well plates (12.5µl sample volume).

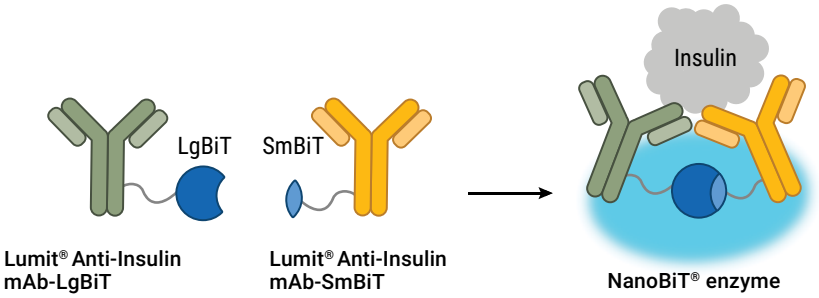


Figure 1. Assay principle. Primary monoclonal antibodies to insulin are labeled with SmBiT and LgBiT. In the presence of insulin, SmBiT and LgBiT are brought into proximity, forming active NanoBiT® enzyme. When Lumit® detection reagent B is added, a bright luminescent signal is generated.

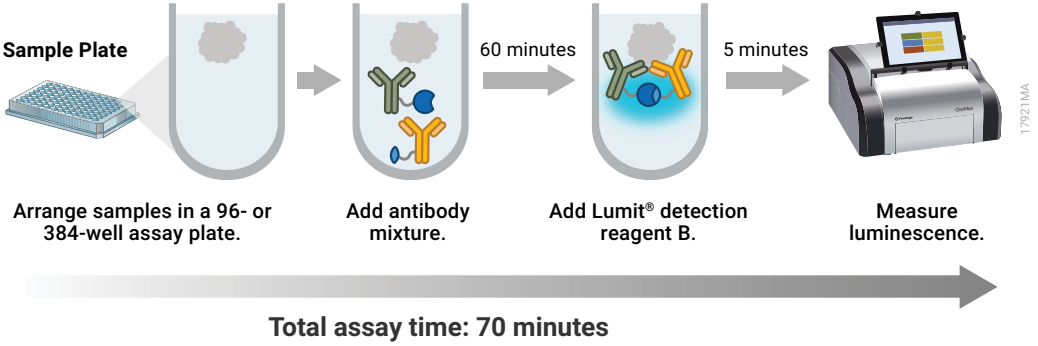


Figure 2. Assay protocol. The Lumit® Insulin Immunoassay is performed using samples containing insulin in a 96- or 384-well assay plate. The Lumit® Immunoassay protocol does not require wash steps and is complete in 70 minutes.

Table 1. Multiwell Assay Formats.

	Insulin Sample Volume	Anti-Insulin Antibody Mix Volume	Lumit® Detection Reagent B Volume	Total Reaction Volume	Recommended Multiwell Plate	Cat.# W8010 Assay Number	Cat.# W8012 Assay Number
	100µl	100µl	50µl	250µl	96-well	50	250*
*	50µl	50µl	25µl	125µl	96-well	100	500
	25µl	25µl	12.5µl	62.5µl	96-well half area	200	1,000
*	12.5µl	12.5µl	6.25µl	31.1µl	96-well half area, 384-well	400	2,000
	10µl	10µl	5µl	25µl	96-well half area, 384-well	500	2,500
	5µl	5µl	2.5µl	12.5µl	384-well, low volume	1,000	5,000

Note: In addition to the formats specified in the kit description (*rows), the assay setup is highly flexible and can be scaled up or down. The assay can be adapted to many sample volumes provided the 1:1:0.5 ratio of Sample Volume: Anti-Insulin Antibody Mix:Lumit® detection reagent B is maintained. See Section 8.A for more information about multiwell plates.

2. Product Components and Storage Conditions

2.A. Product Components

PRODUCT	SIZE	CAT.#
Lumit® Insulin Immunoassay	**100–400 assays**	**W8010**

Sufficient for 100 assays in 96-well plates or 400 assays in 384-well plates. Includes:

- 30µl Lumit® Anti-Insulin mAb-SmBiT, 200X
- 30µl Lumit® Anti-Insulin mAb-LgBiT, 200X
- 5.5ml Lumit® Antibody Dilution Buffer B
- 160µl Lumit® Detection Substrate B
- 3.2ml Lumit® Detection Buffer B

PRODUCT	SIZE	CAT.#
Lumit® Insulin Immunoassay	**500–2,000 assays**	**W8012**

Sufficient for 500 assays in 96-well plates or 2,000 assays in 384-well plates. Includes:

- 5 × 30µl Lumit® Anti-Insulin mAb-SmBiT, 200X
- 5 × 30µl Lumit® Anti-Insulin mAb-LgBiT, 200X
- 5 × 5.5ml Lumit® Antibody Dilution Buffer B
- 5 × 160µl Lumit® Detection Substrate B
- 5 × 3.2ml Lumit® Detection Buffer B

2.B. Storage Conditions

Store all components at –30°C to –10°C, with the following exceptions. After thawing, the Lumit® Antibody Dilution Buffer B can be stored at +2°C to +10°C and the Lumit® Detection Buffer B can be stored at +15°C to +30°C. Do not freeze-thaw components more than three times.

3. Before You Begin

3.A. Preparing an Insulin Positive Control

An insulin positive control can be used to assess the assay performance as well as measure the insulin amount in samples. We recommend preparing a solution of insulin that can be included as a positive control in each experiment. Instructions for preparing and diluting a stock of insulin positive control are provided in Section 4.B.

3.B. Reagent Preparation

The Lumit® Antibody Dilution Buffer B and Lumit® Detection Buffer B must be thawed and equilibrated to room temperature for the experiment. The Lumit® Detection Buffer B can be thawed overnight at room temperature and stored at room temperature once opened. The Lumit® Antibody Dilution Buffer can be thawed overnight at 4°C and then equilibrated to room temperature on the day of the experiment. It can be stored at either +2°C to +10°C or –30°C to –10°C once opened. Please note that the Lumit® Antibody Dilution Buffer B is sensitive to contamination; be careful to maintain reagent sterility.

To conserve components, prepare only the amount of anti-insulin antibody mixture and Lumit® detection reagent B needed for the number of reactions in the experiment. When calculating the amount of anti-insulin antibody mixture and Lumit® detection reagent B needed for the number of samples that will be tested, add some extra (e.g., one to two assays) to compensate for pipetting variability. Also include insulin positive and negative control samples in your calculations. For negative controls, use wells containing only buffer that can be used to measure assay background (background controls).

Prepare the anti-insulin antibody mixture, Lumit® detection reagent B and insulin positive control dilutions fresh on the day of use. Do not store and reuse these preparations.

3.C. Sample Buffer Considerations

The assay is compatible with commonly used PBS and Krebs-Ringer Bicarbonate (KRB) Buffer formulations. KRB often forms the basis for buffers used to collect secreted insulin and glucagon samples in glucose-stimulated insulin secretion (GSIS) or pancreatic islet secretion experiments. These sample buffers typically include BSA. To avoid interference with Lumit® Immunoassay chemistry, the recommended BSA concentrations are 0.1–0.4%, with 0.1% BSA being the preferred concentration. An example sample buffer formulation can be found in Section 8.A.

As the exact buffer composition and BSA concentration will affect the absolute value of the relative light units (RLU), all samples and dilutions of the insulin positive control should be prepared in the same buffer.

Use personal protective equipment and follow your institution's safety guidelines and disposal requirements when working with biohazardous materials such as cells and cell culture reagents.

3.D. Materials to Be Supplied by the User

- insulin, as a positive control (e.g., MilliporeSigma™ Cat.# 91077C, human recombinant insulin)
- white, multiwell assay plates compatible with a luminometer (solid white or white with clear bottom; also see Section 8.A)
- multichannel pipette
- pipette tips, preferably with aerosol filters
- multichamber, dilution reservoir (e.g., Dilux® Cat.# D-1002) or tubes for dilutions
- reagent reservoir trays (e.g., Thermo Fisher Scientific™ Cat.# 8095)
- plate shaker for mixing multiwell plates
- luminometer capable of reading multiwell plates (e.g., GloMax® Discover System, Cat.# GM3000)

4. Protocol for Measuring Insulin

This protocol describes the analysis of samples containing insulin in multiwell assay plates based on an insulin sample volume of 50µl assayed in a 96-well plate. For this sample volume, the reaction includes: 50µl of insulin sample + 50µl of antibody mixture + 25µl of Lumit® detection reagent B.

However, other sample volumes and plates can be used if the 1:1:0.5 volume ratio of insulin sample:antibody mixture:Lumit® detection reagent B is maintained, e.g., 12.5µl + 12.5µl + 6.25µl in 384-well plates. The volumes of antibody mixture and Lumit® detection reagent B needed for additional sample volumes can be found in Table 1.

4.A. Preparing Samples

This assay has been developed for use with cell culture samples to measure insulin secretion from cell lines or islets. It detects both human and rodent (mouse and rat) insulins (see Section 6). Assay performance with additional sample types must be determined by the user. This assay is not recommended for use with blood-based samples (i.e., whole blood, plasma and serum).

1. Collect insulin samples in a buffer compatible with the assay (see Sample Buffer Considerations above).
2. Dilute samples into the assay range (10pM–8nM) if needed.

4.B. Preparing and Diluting the Insulin Positive Control

An insulin positive control can be used to assess the assay performance as well as measure the insulin amount in samples. Before beginning experiments, a stock of insulin positive control can be prepared, aliquoted and frozen. We prepare a 1mM stock of insulin using commercially available insulin powder (e.g., Sigma Cat.# 91077C, human recombinant insulin). Weigh out >10mg insulin powder. Resuspend insulin in 0.01N HCl by adding 1ml of 0.01N HCl per 5.8mg of insulin. Use immediately or dispense into tubes (e.g., 25–50µl per tube) and store following the manufacturer's instructions.

To measure the amount of insulin in samples, prepare a titration curve using the insulin positive control. The insulin should be diluted to at least 8nM to establish the upper limit of the titration curve. Two- or threefold serial dilutions can then be prepared for an 8- or 12-point curve. This protocol is for generating a 12-point curve with twofold dilutions starting with a 1mM stock of insulin. Representative data are shown in Figures 4 and 5.

As different buffers and their components, such as BSA, can affect light output, it is important that the insulin dilutions be prepared in the same buffer as the samples.

Wells containing only buffer should be included in all experiments as background controls. These wells are used to measure the assay background signal and to calculate the signal-to-background and signal-to-noise ratios.

1. If previously prepared and frozen, thaw the insulin positive control immediately before use.
2. Briefly centrifuge the tube to collect all contents at the bottom of the tube before opening.
3. Mix by gently vortexing.

4. Prepare an initial concentration of 10µM insulin by diluting the insulin positive control 100-fold into the same buffer solution as the test samples. Follow this with an additional 100-fold dilution and 12.5-fold dilution. Recommended dilution volumes are below and in Figure 3.

   a. Prepare 10µM insulin by adding 10µl of 1mM insulin positive control to 990µl of buffer.

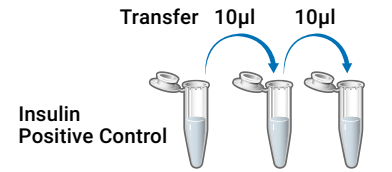
   b. Prepare 100nM insulin by adding 10µl of 10µM insulin to 990µl of buffer.

   c. Prepare 8nM insulin by adding 80µl of 100nM insulin to 920µl of buffer.

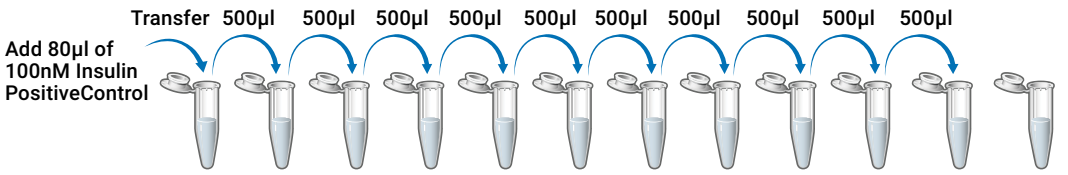
   Note: Mix thoroughly after each dilution. Change pipette tips between each dilution to avoid insulin carryover and use aerosol filter tips. The range of the assay is large, so carryover from high to low concentrations can compromise the dilution series linearity.

5. Continue to prepare ten twofold serial dilutions of the insulin in tubes or a multichamber dilution reservoir. The last tube or chamber should contain only buffer for the background control.

   Note: Mix each dilution thoroughly before moving to the next tube or chamber. Be careful not to contaminate the background control with insulin.



Buffer Volume		990µl	990µl
Insulin (µM)	1,000	10	0.1
Insulin (nM)	1,00,000	10,000	100



Buffer Volume	920µl	500µl	500µl	500µl	500µl	500µl	500µl	500µl	500µl	500µl	500µl
Insulin (nM)	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.0625	0.0313	0.0156	0.0078	0
Insulin (pM)	8,000	4,000	2,000	1,000	500	250	125	62.5	31.2	15.6	7.8	0

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Figure 3. Insulin positive control dilution series.

4.C. Adding the Samples

1. Add 50µl of each insulin experimental sample to the wells of a 96-well plate.

2. Add 50µl of each insulin positive control dilution and background control to the wells of the 96-well plate.

   Note: We recommend assaying each sample, dilution and background control in duplicate or triplicate. Incorporate assay samples and controls on the same plate for normalization.

4.D. Adding the Anti-Insulin Antibody Mixture

After adding samples and controls to the assay plate, prepare the anti-insulin antibody mixture.

1. Use the Lumit® Antibody Dilution Buffer B that has been thawed and equilibrated to room temperature (see Section 3.B, Reagent Preparation).
2. Immediately before use, remove the Lumit® Anti-Insulin mAb-SmBiT and Lumit® Anti-Insulin mAb-LgBiT antibodies from –30°C to –10°C.
3. Briefly centrifuge the tubes to collect all contents at the bottom of the tube before opening.
4. Gently vortex the tubes to mix.
5. Immediately prior to use, prepare the anti-insulin antibody mixture by making a 200-fold dilution of each antibody into the Lumit® Antibody Dilution Buffer B (i.e., 5µl of each antibody to 1ml of buffer). Calculate the amount of mixture needed for the experiment, including extra for pipetting variability, using the table below.

Reagent	Volume per Well	Volume per 100 Wells
Lumit® Antibody Dilution Buffer B	50µl	5ml
Lumit® Anti-Insulin mAb-SmBiT	0.25µl	25µl
Lumit® Anti-Insulin mAb-LgBiT	0.25µl	25µl

6. Thoroughly mix the anti-insulin antibody mixture by gently vortexing.
7. Add 50µl of the antibody mixture to the wells containing insulin test samples, background controls and positive controls.
8. Gently mix the plate (e.g., 20 seconds on a plate shaker at 250–350rpm).
9. Incubate for 60 minutes at room temperature.

4.E. Adding Lumit® Detection Reagent B

Prepare the Lumit® detection reagent B while the samples are incubating with the anti-insulin antibody mixture (Section 4.D). Begin preparations approximately 10 minutes before the end of the incubation period.

1. Use the Lumit® Detection Buffer B that has been thawed and equilibrated to room temperature (see Section 3.B, Reagent Preparation).
2. Remove the Lumit® Detection Substrate B from –30°C to –10°C storage, mix and briefly centrifuge.

3. Prepare Lumit® detection reagent B by making a 20-fold dilution of Lumit® Detection Substrate B in Lumit® Detection Buffer B. Prepare enough volume of Lumit® detection reagent B for the number of wells to be assayed.

Reagent	Volume per Well	Volume per 100 Wells
Lumit® Detection Buffer B	23.75µl	2.375ml
Lumit® Detection Substrate B	1.25µl	125µl
Total Volume	25µl	2.5ml

Note: Prepare the reagent just before it is needed. Once reconstituted, Lumit® detection reagent B will lose 10% activity in approximately 3 hours at +10°C to +30°C.

4. Transfer the Lumit® detection reagent B to a reagent reservoir tray for easy pipetting.
5. Add 25µl of Lumit® detection reagent B to each well.

   Note: Work quickly and efficiently using a multichannel pipette to minimize variability from well-to-well and across the plate.
6. Gently mix the plate (e.g., 20 seconds on a plate shaker at 250–350 rpm).
7. Incubate 3–5 minutes at room temperature.
8. Read luminescence.

   Note: If there is more than one plate in the experiment, add the Lumit® detection reagent B to the first plate and read that plate before adding reagent to the second plate. Continue in this way until all plates have been read. For more information and options, see Signal Stability in Section 8.A, General Considerations.

5. Assay Controls and Data Analysis

Assay performance can be evaluated using positive controls prepared from the insulin positive control and background controls consisting of wells containing buffer only. For this analysis, an insulin dilution series is prepared and each concentration is assayed in duplicate or triplicate. The data from replicate wells is averaged and plotted. The data are used to define the linear range of the assay and calculate parameters such as signal-to-background ratios, signal-to-noise ratios and sensitivity.

When signals from experimental samples fall within the linear range of the assay, there is a linear relationship between RLU and insulin concentration, and samples can be directly compared. Results can be described simply in terms of RLU or a sample-to-sample ratio, such as “fold increased insulin secretion” calculated from a treated and untreated sample. When working with signals close to the background control, first subtract the background control signal from all samples.

Insulin concentration in experimental samples can be calculated using the insulin positive control titration curve. When planning the dilution series, select concentrations that encompass the range of your experimental samples. Each dilution should be assayed in duplicate or triplicate. The signals from replicate wells are averaged and plotted. The graph can then be used to estimate insulin concentration in experimental samples.

6. Representative Data

6.A. Assay Performance

Titration of Insulin Positive Control

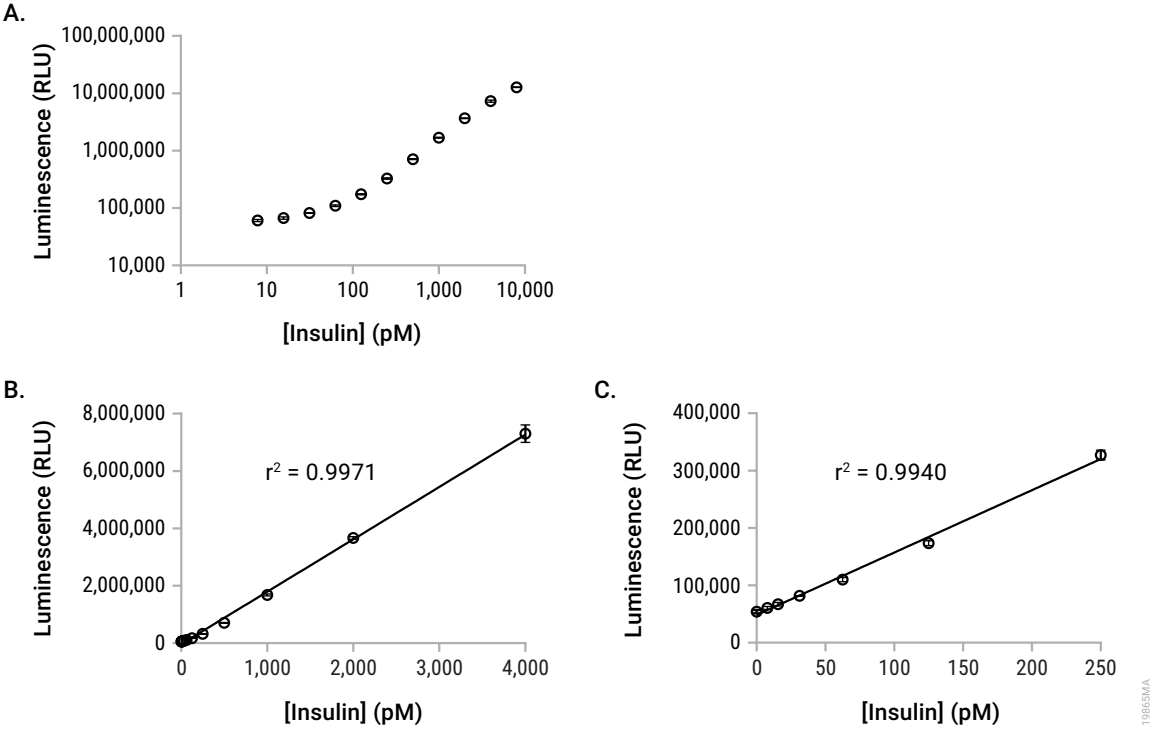

Figure 4. Titration curve for the Lumit® Insulin Immunoassay. An insulin positive control was prepared following the instructions in Section 4.B (using human recombinant insulin, Sigma Cat.# 91077C) and serially diluted in PBS buffer + 0.1% BSA. Fifty microliters of each concentration was assayed in quadruplicate in 96-well plates. The luminescence (RLU) was recorded using a GloMax® Discover System. The average value of the replicates is plotted. Error bars are ± 1 standard deviation. The percent CV was ≤5%. **Panel A.** Log-log plot, starting with the 8nM dilution. **Panel B.** Linear-linear plot, starting with the 4nM dilution. **Panel C.** Linear-linear plot of data points near the 0pM background control.

Note: These are representative titration curves. Absolute RLU values vary based on many experimental factors. Therefore, they should not be used for interpolation of unknowns. Generate a titration curve for each experiment to interpolate experimental samples.

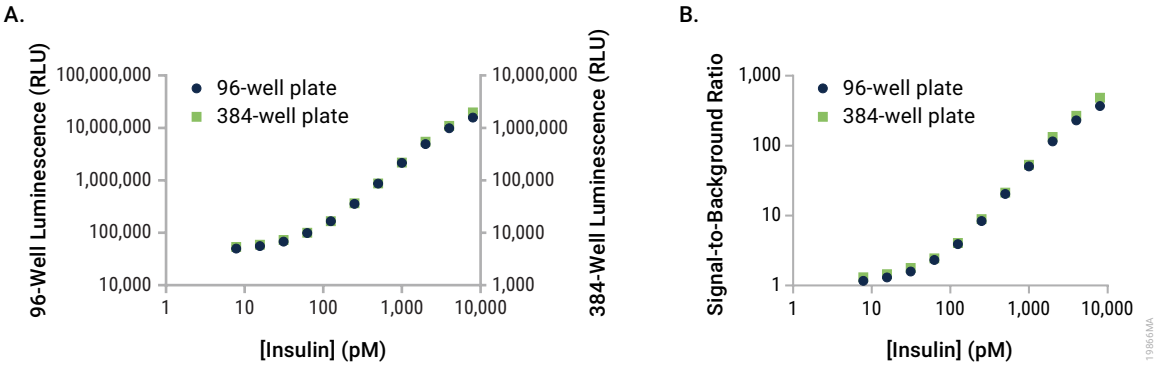

Figure 5. Lumit® Insulin Immunoassay compatibility with lower assay volumes. An insulin positive control was prepared following the instructions in Section 4.B and serially diluted in PBS Buffer + 0.1% BSA. The positive control dilutions were assayed in quadruplicate in either 96- or 384-well plates using 50µl for 96-well plates and 12.5µl for 384-well plates. The luminescence (RLU) was recorded using a GloMax® Discover System. The average value of the replicates was calculated. **Panel A.** Log-log plot of the average values. Error bars are ± 1 standard deviation. **Panel B.** Log-log plot of the signal-to-background ratio, calculated by dividing the average signal for each dilution by the average signal of the background control (0pM).

6.B. Example Data with Biological Samples

Glucose-Stimulated Insulin Secretion in Cultured Cells

Beta cell function can be studied in vitro using islets and cell lines. However, only a few cell lines have been identified as being responsive to glucose in culture, including the INS-1 and INS-1E rat cell lines and MIN-6 mouse cell line (5).

Insulin secretion in response to glucose treatment is studied in glucose-stimulated-insulin secretion (GSIS) experiments. Typically, cells are plated in 24- or 96-well plates. (The number of cells plated per well for optimal glucose response needs to be empirically determined.) After culturing, the cells are washed and then challenged with multiple glucose concentrations, possibly in combination with other treatments, to induce insulin release. Samples of the supernatant are collected for insulin measurements. Only an aliquot of insulin sample is needed, which can be transferred to 96- or 384-well plates and assayed with the Lumit® Insulin Immunoassay.

Perifusion of Mouse Islets

Perifusion is a powerful method for studying insulin and glucagon secretion over time and in response to sequential treatments. The time-resolved data provides important insights into islet function. Samples are collected frequently (e.g., every minute) over an extended time (e.g., >1 hour). To obtain the most useful information from the data set, assay all samples. However, the large sample number can make this prohibitive when using traditional ELISA methods. The Lumit® Immunoassay solution-based approach can facilitate analysis. Many samples can be assayed quickly and in 384-well formats. In addition, because minimal sample volumes are needed, collected samples can be split into two wells to assay for both insulin and glucagon using the Lumit® Insulin and Lumit® Glucagon Immunoassay, respectively. For more information on Lumit® Immunoassays, visit: **www.promega.com/products/immunoassay-elisa/lumit-immunoassays/metabolic-target-research-with-lumit-immunoassays/**

6.B. Example Data with Biological Samples (continued)

An example of using Lumit® Insulin and Lumit® Glucagon Immunoassays to test mouse islet perifusion samples is shown in Figure 6. The same samples were analyzed for both hormones, allowing the two data sets to be superimposed.

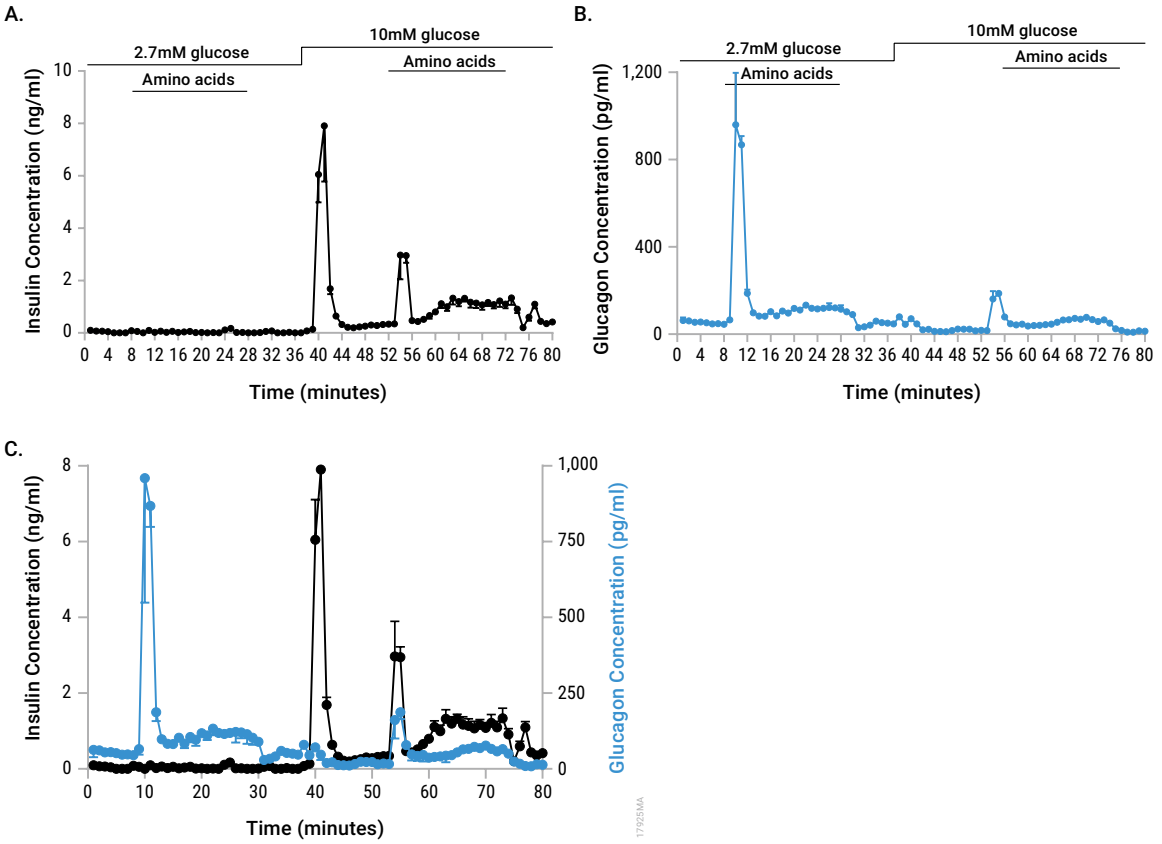

Figure 6. Perifusion experiments: Measuring insulin and glucagon. Insulin and glucagon secretion were measured in samples collected during perifusion experiments. This data was kindly provided by Drs. Hannah Foster and Matthew Merrins, University of Wisconsin VA Hospital, Madison, WI. The samples were collected according to published protocols (6). Briefly, 80 mouse islets were placed in triplicate chambers of a perifusion instrument (Biorep, Miami Lakes, Florida). The islets were treated with 2.7mM glucose and then 10mM glucose in combination with an amino acid mixture. Perifusate (100µl) was collected every minute. Ten microliters of each sample were transferred into wells of a 384-well plate and assayed for either insulin (**Panel A**) or glucagon (**Panel B**). **Panel C.** The superimposed data sets. The data is the average of triplicate chambers and the error bars are plus or minus 1 standard deviation.

7. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. Email: **techserv@promega.com**

Symptom	Causes and Comments
RLUs are not identical to those in this Technical Manual	Bioluminescent signal intensity, e.g., absolute RLU, will vary between laboratories due to several factors, such as specific experimental conditions (buffers, volumes) as well as plates and plate readers. Establish the assay performance in your lab with the sample buffer, plates and instruments you will be using. An insulin positive control can be used to generate a titration curve that will help establish the parameters expected in your lab. Range and signal-to-background ratio are some parameters that can be optimized.
Luminescence for the titration curve is variable between experiments	There may be some variation in absolute RLU due to experimental conditions, such as specific buffers used, temperature, etc. Include a titration curve for each experiment, run under the same conditions as the test samples, to measure insulin concentrations. Though the RLU may vary, the assay range and signal-to-background should still be comparable between experiments.
The insulin titration curve is not linear	Mix each dilution thoroughly before moving on to the next tube or chamber. The dilutions should be carefully prepared, avoiding carryover from a higher concentration. The sensitivity of the assay and the broad range (~3 logs) means that any carryover will compromise the linearity of the dilution series. Change pipette tips after each dilution step. Also, make sure that no insulin contaminates the background control.
Dilutions at higher concentrations have lower light units than dilutions at lower concentrations	The dilutions at higher concentrations are above the range of the assay and need to be further diluted. This is due to the "hook effect," which occurs when excess antigen prevents both antibodies from binding to a single antigen thereby preventing the LgBiT and SmBiT from complementing each other.
High background observed	Background can be increased by the spurious association of the antibodies over time. Another cause of high background is insulin contamination of the buffer or background control wells. Avoid contamination by changing tips often and preparing the insulin dilution series separately from the Anti-Insulin Antibody Mixture. Prepare the antibody mixture right before use to prevent elevated background.

7. Troubleshooting (continued)

Symptom	Causes and Comments
Greatly reduced assay sensitivity when applied to serum samples	Several factors within serum samples can interfere with the homogeneous Lumit® Insulin Immunoassay performance and, thereby, significantly reduce assay sensitivity. For this reason, we do not recommend using the Lumit® Insulin Immunoassay with any blood-based samples, including serum.

8. Appendix

8.A. General Considerations

Sample Buffer Formulations

This is an example of a buffer that is compatible with the Lumit® Insulin Immunoassay and can be used in GSIS experiments:

Sample Buffer

136mM	NaCl
4.7mM	KCl
1.2mM	$MgSO_4$
1mM	$CaCl_2$
1.2mM	KH_2PO_4
5mM	$NaHCO_3$
10mM	HEPES (pH 7.5)
0.1%	BSA

Species Specificity

The antibodies used in the Lumit® Insulin Immunoassay recognize human, mouse and rat insulin.

Cross-Reactivity

Cross-reactivity with proinsulin and C-peptide were determined. All were tested at a range of concentrations up to 8nM and the percent of insulin light signal was calculated.

Peptide	Insulin Signal Percent
Proinsulin (human)	30%
C-peptide (human)	Not detectable over background

Signal Stability

After the addition of the Lumit® detection reagent B, the light signal is stable with a half-life of approximately 1 hour. There are two approaches to read multiple assay plates to allow for plate-to-plate light signal comparisons.

One approach, described in Section 4.E, is to add the Lumit® detection reagent B to one plate at a time, reading each plate after a 3–5 minute incubation before moving on to the next plate. This sequential processing ensures consistent light units between plates.

The second approach is to add the Lumit® detection reagent B to all assay plates at once. The 1-hour signal half-life is compatible with this batch processing of multiple plates. To control for any decrease in light signal during the time it takes to read multiple plates, we recommend incorporating positive controls on each assay plate for normalization.

Temperature

The intensity and stability of the luminescent signal is temperature sensitive. For consistent results, equilibrate sample buffers, Lumit® Antibody Dilution Buffer and Lumit® Detection Buffer B to room temperature before use. Insufficient equilibration may result in increased well-to-well variability across the plate.

Assay Plates and Equipment

Use opaque, white-walled multiwell plates for the bioluminescent assay. Light signal is diminished in black plates. Increased well-to-well crosstalk is observed in clear plates.

The assay is compatible with various multiwell plate formats (96- and 384-well; Table 1) and multiple sample volumes (5–100µl; Table 1). Examples of plates are below. Choose plates that are compatible with your luminometer.

Plate Format	Plate Catalog Numbers
96-well	Corning® Cat.# 3912
96-well, half-area	Corning® Cat.# 3693
384-well, regular volume	Corning® Cat.# 3572
384-well, low volume	Corning® Cat.# 4512

All standard plate readers capable of reading luminescence are suitable for this assay. An integration time of 0.25–1 second per well should be suitable. Some instruments might require optimizing the gain settings to achieve sensitivity and dynamic range. Consult the instrument manual for instrument settings.

The GloMax® Discover Microplate Reader has a pre-installed “Lumit Immunoassay” protocol under the ‘Protocols’ tab that can be used. It has a 0.5 second integration time.

Note: The light signal values will vary depending on the specific plates and luminometers used to generate the data.

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8.B. References

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[a]U.S. Pat. Nos. 9,797,889, 9,797,890, 10,107,800 and 11,493,504; European Pat. No. 2970412; Japanese Pat. Nos. 7280842 and 7532562; and other patents and patents pending.

[b]U.S. Pat. No. 8,809,529, European Pat. No. 2635582, Japanese Pat. No. 5889910 and other patents and patents pending.